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# An Investigation of electrophoresis gel silver staining using large area sample inclusive polymerization

Lloyd C. Litt

# AN INVESTIGATION OF ELECTROPHORESIS GEL SILVER STAINING USING LARGE AREA SAMPLE INCLUSIVE POLYMERIZATION

by

Lloyd C. Litt

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in the Center for
Imaging Science in the College of
Graphic Arts and Photography of the
Rochester Institute of Technology

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CERTIFICATE OF APPROVAL

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M.S. DEGREE THESIS

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The M.S. Degree Thesis of Lloyd C. Litt
has been examined and approved
by the thesis committee as satisfactory
for the thesis requirement for the
Master of Science degree

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## ABSTRACT

An electrophoresis silver stain was investigated using a unique method of sample introduction in polyacrylamide gel media. A protein inclusive polymerization technique is described that produces large area gels with controlled protein concentration steps.

Using gels with Bovine Serum Albumin sample steps,an organic dye stain, Coomassie Brilliant Blue was found to be extremely linear in stain response. When used on a published silver stain (Merril,et. al.) a non-linearity caused by a diffuse background density was observed.

# DEDICATION

This thesis is dedicated to my parents,
my wife,Judy,
and the memory of Dr. Ronald Francis.
Each of these people contributed in
their own way to the
completion of this work.

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# INTRODUCTION

Photochemical technology has found application in many fields, ranging from the very sophisticated (the fabrication of multilayered semiconductor chips) to consumer products (autodarkening glasses). In laboratory research, one very interesting use has been the utilization of a silver deposition process ("silver staining") to improve the limit of detection of biological compounds separated by electrophoresis techniques. These silver stains use chemical processes that are very similar to those used in conventional silver halide photographic systems.

This dissertation describes experimentation designed to assess the photochemistry of the silver staining process using a unique method of sample introduction.

## A. Investigation Objectives

The primary goal of the investigation was to study the feasibility of developing an improved silver stain which would provide increased sensitivity. The impetus for this was the concept that the combination of chromogenic color development chemistry with essentially known silver staining techniques would result in increased density and thereby effect a great reduction in detection limits of electrophoresis visualization systems.

While systems employing chromogenic dye formation chemistry for photography are significantly more sophisticated than electrophoresis staining schemes, the specific limitations on dye chariacteristics such as color fidelity, dye stability and performance would not be factors in silver staining. A summary of basic chromgenic devlopment systems used in photography is presented in Appendix B of this thesis.

The initiation of a program to develop an dye-additive stain begins with an investigation of recently published silver stains, familiarization with the electrophoretic process and, most critcally, the development of techniques to allow quantitative assessment of the effect of the numerous variables involved.

This thesis describes the work done to begin development of a dye-additive silver stain. As will become apparent in the sections that follow, logistical and practical issues restricted the overall program to familiarization and investigatory phases; however, the development and validation of a novel method for the study of stain characteristic responses was completed. The use of this technique should greatly facilitate future research to produce ultrasensitive stains combining the deposition of silver metal with the chemical formation of high density dyes.

B. Silver Halide Image Formation

Conventional silver halide photographic systems make use of several types of chemical processes to record and amplify an image. In general, light sensitive silver halide crystals form small silver metal 'latent image' sites when exposed to a sufficient amount of actinic radiation. These 'latent image' sites are not directly visible but act as a key factor in the selectivity of a chemical amplification process.

The chemical amplification of a small silver 'latent image' into a large silver metal structure can be accomplished in several ways. Generally, most systems use a chemical reduction process to convert silver ions ($Ag^+$) into a neutral, stable silver metal ($Ag^0$). Using chemical reduction, a single latent image containing as few as $4Ag^0$ atoms can be amplified into a massive silver structure containing many thousand atoms and may grow as large as several hundred nanometers.[1]

There are several subcategories of chemical development that differ mainly in the source of the $Ag^+$ ions used to form the silver metal image. "Direct Development" uses a chemical reducing agent in solution which converts the silver ions contained in a silver halide crystal into the silver metal image. "Prefixation Physical Development" refers to systems containing both a reducing agent and silver ions in the

developing solution; in this case the silver halide crystals remain but are not the source of the silver ions used in the image formation. "Postfixation Physical Development" uses silver ions from the developing solution but the silver halides have previously been removed. In the case of "Solution Physical Development", the silver ions are dissolved from the silver halides into the developer solution and then used in the development process.[2]

C. Electrophoresis and Visualization

Electrophoresis (EP) has found widespread laboratory use as a high resolution separation technique for research, analysis and clinical diagnostics. Various configurations of the method find primary application in the separation of proteins, nucleic acids and enzymes, as well as many other organic molecules. While early electrophoretic methods were solution based, limited resolution due to convection currents and heat dissipation, coupled with a lack of detection sensitivity and relatively difficult sample handling, have led to systems employing a rigid, non-convective, porous media as a basic structural network. Gels formed with agarose and polyacrylamide have proven to be by far the most useful and popular. Various versions are now readily available in a number of commercial forms and these systems have greatly increased the resolution and applications of modern EP

separations. Appendix A is a broad description of the basic technology of gel electrophoresis.

Since the vast majority of materials separated by electrophoresis are either colorless or of insufficient concentration to be directly detected, a number of procedures are in common use for the visualization and/or quantification of the separated species. The most direct approach, and in widespread use for protein detection, involves soaking the gel in a dye solution to stain the sample molecules in the gel by coulombic attraction or acid/base salt reactions. These dye staining methods (often adapted from histological systems) rely principally on the amphoteric nature of the proteins and the charges of the dye molecules in solution.[3] Acidic and basic dyes can be chosen to react with the proteins according to the general reactions below to form salts and/or proteinates.

$$PROTEIN^{+} + DYE^{-} \text{ ------> } (PROTEIN^{+}\ DYE^{-})$$

or

$$PROTEIN^{-} + DYE^{+} \text{ ------> } (PROTEIN^{-}\ DYE^{+})$$

Since proteins are very complex and contain both carboxyl (acidic) and amino (basic) groups, both positively or negatively charged dyes will react to varying degrees depending on the structure of the protein and the pH of the sample solution. A major application criteria for the choice of dye is therefore dependent on the pH relative to the isoelectric point of the protein.

Unfortunately, dye staining methods often exhibit inadequate sensitivity for many modern EP uses. The effective detection of samples is often severely limited by the molar absorptivity and, to some extent, molecular weight of most dyes; the most common dyes in use for histological or EP staining have molecular weights up to several thousand grams per mole. This relatively large size restricts diffusion into the gel matrix. This also means that the speed of destaining, elimination of unwanted background, will also be very slow. This is especially of concern when the affinity of the dye for the protein is low. In this case, destaining may remove color from the sample by dissociation during the extended destaining procedure. In addition, if a sample of many different protein-like species is to be visualized (the usual case), the effective staining power will vary greatly from molecule to molecule and direct relationships between density formed and sample concentration generally can not be assumed.

Another very popular method of visualizing samples is autoradiography: the exposure of photographic film by radioactive emissions contained in the gel. This method requires that the separated samples either contain a radioactive emitter or can be readily derivatized by reaction with a radiolabel after separation. Suitable radionuclides for direct application include $^{131}I$, $^{59}Fe$, $^{125}I$ and $^{32}P$. As they are likely to be incorporated directly into the biomolecule, low energy emitting radioisotopes such as $^{14}C$,$^{3}H$, $^{35}S$ are very widely used; often, however, combination with a fluor impregnated into the gel is necessary. In this case, the radioactive emissions can excite the fluor which emits light to expose the photosensitive emulsion. With either category of radiolabels, the completed gel is brought into intimate contact with a sheet of X-ray film for exposure. The exposure is determined by the strength of the isotope used,the concentration of the isotope in the sample, the duration of the contact exposure, any quenching that may occur and by the sensitivity of the film-developer system.

Autoradiography, although capable of producing high sensitivity maps of a separation, has several major limitations. Obviously, if the original sample is not labeled with a radioactive isotope, such a procedure is either not applicable or requires a relatively hazardous post-separation derivatization to be done. In addition, the resolution of the

autoradiograph may be limited by the spreading of the radiation as it passes from the gel to the film and by the strength of the emissions.

Often, autoradiography is used in combination with other staining methods. The amount of radioactivity, and therefore the approximate exposure duration, can be inferred from the amount of visual density formed in a pre-exposure staining treatment. This procedure is often based on educated guesses and is not standardizable due to the numerous variables involved. Certainly the strongest deterrent factor in autoradiography is the use of radioactivity itself; most clinical labs are not equipped for the special requirements needed to safely handle 'hot' samples and although this is not generally true in research, problems with radioactive waste have similarly retarded use in these labs.

Other visualization methods include fluorographic tagging and direct UV scanning. Fluorographic tagging is similar in many respects to autoradiography and has the advantage of not being as hazardous. However, this method requires careful selection of fluorophor to obtain uniform reactivity with the sample species. Direct UV scanning does not require special staining treatments because it relies upon the UV absorption of organic compounds. This method is severely limited by the transmittance of the support media used (polyacrylamide has

a steep increase in absorption at approximately 250 nanometers)[4] as well as the relatively low molar absorptivity of most biological molecules. In work with nucleic acids, fluorescent dyes such as ethidium bromide which 'intercalates' between the DNA strands, are of great use. Many of these, however, are either carcinogenic or mutagenic.

## D. Silver Staining

As is clear from the discussion above, there has been a strong need for development of a generally applicable, inexpensive, simple and highly sensitive method of gel visualization.

Modern silver stain methods are the result of an evolutionary process that can be traced back to early histological stains used in the 1800's by biological researchers such as Krause, Golgi and Cajal.[5] In 1963, Fredrick adapted a histological silver stain for use on proteins separated by electrophoresis.[6] The procedure has been widely modified, applied and improved and now represents a very basic technique. Figure I, showing only papers where silver staining appears in the title or keywords, demonstrates the speed of acceptance of the technique by the scientific community.

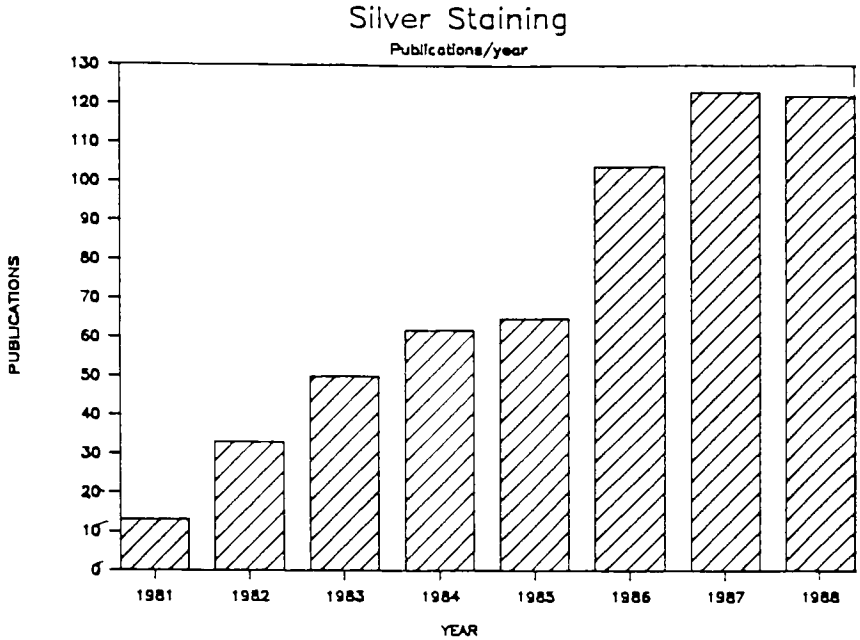



Figure 1. Silver Staining Publications

As with many dye staining techniques, the silver stains are largely adaptations of existing histological staining procedures. There are several basic silver stains that are used routinely in histological tissue staining for analysis by microscopy. The earliest versions yielded a negative image and used silver nitrate solutions followed by a photographic developer to produce dark background with light 'images'.[7] The low sensitivity of these systems gave rise to multistep photochemical reversal systems. The actual staining steps vary only slightly among different methods. After fixation, the procedures include treatments in a bleach or photo-reversal bath as well as a silver nitrate solution. A carbonate or formaldehyde/carbonate treatment may precede or

follow the silver nitrate treatment. The most recent stains place carbonate/formaldehyde bath following the silver treatments. Development is stopped by a wash or acid rinse treatment.[8]

The first step in all silver staining procedures is preparative fixation of the samples in the gel following separation. The primary purpose of fixation is to prevent leaching of the sample molecules from the gel. Several common methods include treatments with oxides of heavy metals, glutaraldehyde, methanol, acetic acid or trichloroacetic acid.[9] After the samples have been fixed in the gel, a photo-reversal 'bleach' step is used. Many conventional photographic bleaches appear among those used in silver staining, notably dichromate, ferricyanide, and permanganate.[10] Merril, et. al.[11], claims that dichromate is preferred for sensitivity. Following photo-reversal, a system of latent image formation is required. Early methods used a two bath development, first with a base such as sodium carbonate then with ammoniacal silver solution with formaldehyde and tungstosilicic acid.[12] Later Merril et. al.[13], Oakley et. al.[14] ,and others[15] simplified and modified the procedure. The new methods used a bath of ammoniacal silver prior to a citric acid-formaldehyde developer bath. It seems obvious that only the silver trapped in the gel is available to form density when placed in the developer solution.

The actual mechanism of silver/sample interaction is mostly uncertain and is a current subject of considerable research. The complexity of the protein or nucleic acid samples makes investigation difficult due to the large number of molecular substituents present. It is usually inferred that the samples interact with the silver ions to produce a chemical latent image' and this latent image may then act as a nucleation site for physical silver metal conversion through the action of a developing agent.[16] Several papers describe treatments with aldehydes such as formaldehyde or glutaraldehyde; it has been suggested that these treatments act as either mild oxidants, react to cross-link the sample molecules or provide free aldehyde groups for reaction with silver ions in solution.[17] It is generally known that silver is readily complexed by several chemical groups and the selectivity of certain staining methods has led to speculation dealing with this silver sample interaction. Several researchers indicate the possibility for carboxyl[18] or sulfhydryl[19] groups as key in the silver-sample interactions.

Early studies of silver and silver complexes date back to the 1880's when Prescott[20] began studies of the complexation of silver with ammonia in solution. Similar studies of these silver complex reactions were later by others until, in 1933, Britton and Wilson[21] continued the study and

were able to determine the equilibrium equations for ammoniacal silver nitrate solutions. It is theorized that these relationships determine the free silver ion concentration in ammoniacal silver stains. The composition of silver stains places the equilibrium such that the free silver ion concentration is very low.[22] Ammoniacal silver stains had several drawbacks. The staining procedures required many handling steps and used large amounts of expensive silver nitrate.[23] Handling of the gels will cause dark artifacts when these (or other) silver stains are used.[24] The stains also produced a dark brown background which limited the sensitivity.[25] Unwanted background coloration can be removed by treatment of the stained gel with photographic fixer solutions containing sodium thiosulfate[26] but this method is inadequate because destaining occurs in the image bands as well as in the background.

Several procedures have been developed that reduce background darkening, increase sensitivity, and simplify the process steps. These systems consist of an alcohol-acid fixation, glutaraldehyde cross-linking, ammoniacal silver stain, and citric acid-formaldehyde developer.[27] It has been found that washing of the gels and cleanliness of the reaction environment is very important in the reduction of unwanted background stains.[28,29] The stained image can be intensified by recycling with fresh silver nitrate followed by

carbonate/formaldehyde.[30] Over-staining or recycling will cause background density to increase.

## E. Silver Halide Systems/Silver Stains

As can be seen in Table 1, there are numerous similarities between modern silver stains used on electrophoresis gels and conventional silver halide photographic systems.

TABLE 1 - Comparison of Silver Stain To Silver Halide Systems

| | EP STAIN | AgX |
|---|---|---|
| ENVIRONMENT | Polyacrylamide (or Agarose,etc.) | Gelatin |
| Ag SOURCE | $AgNO_3$ solution | AgX crystals |
| LATENT IMAGE | Sample molecules | $Ag^0$ sites |
| IMAGE REVERSAL | Chemical Oxidizer (Ferricyanide,etc.) | Chemical Oxidizer (Ferricyanide,etc.) |
| DEVELOPER | Chemical reducer (Formaldehyde) | Chemical reducer (HQ,Metol,etc.) |

# METHODS

Due to the unique nature of this thesis project, many special supplies were required. These objects were either purchased from manufacturers or fabricated in the lab. Several of these devices were not actually used in the thesis due to various changes in requirements of the project.

## A. General Handling Techniques:

In order to produce successful gel and stain results, procedures were developed over the course of the study to optimize the handling of each step. The sensitivity of silver stains necessitated special care in the handling of gels and cleaning of all glassware. Procedure for cleaning of glassware and gel mold plates can be found in Appendix X.

All solutions were carefully prepared using fresh distilled water obtained from Nalgene reservoir containers located in the Imaging Science Chemistry Lab.

In an effort to reduce contamination of experimental solutions or gels by contact with skin surfaces, any handling of gel material and plates during polymerization or staining was done using PVC gloves. A distilled water rinse was

employed just prior to use in order to clean any contaminants, such as talc which is provided on glove interiors, from the exterior of the gloves. Many of the chemicals used to make polyacrylamide gels are toxic or irritating substances. For this reason gloves were also used in order to protect the skin of the researcher's hands.

B. Gel Hardware:

In order to study electrophoresis silver stains, it was necessary to fabricate many gels containing known amounts of sample for study. A system for molding gel sheets was assembled consisting of glass plates and spacers. Four 4" x 5" glass plates, four 4" x 10"glass plates, and six .5" x 10" x 1 mm plastic spacers were purchased from Dan Kar Inc., Reading, MA. Gels were molded using two plates separated on three sides by spacers. The spacers were coated lightly with Vaseline brand petroleum jelly in order to improve the seal formed on the edges and prevent leakage of the gel solution from the mold.

A simple electrophoresis cell was fabricated from scrap pieces of clear PLEXIGLAS. The pieces were cut to shape and then glued with epoxy and the joints of the buffer reservoirs were coated with silicone sealant to prevent leakage of buffer solution.

It was later determined that actual electrophoresis of samples would not only be inconvenient but would not allow easy quantitative evaluation of the stain. It was hoped that the apparatus could still be of use as a gel casting stand. Several gels were cast by placing the glass plate/spacer assembly in the apparatus vertically such that gel solution could be poured into the open top of the mold. Unfortunately, the apparatus did not provide clamping to the lower portions of the mold assembly. This allowed the plates to separate at the bottom and the gel solution would leak from the mold before polymerization could occur.

A new method of gel casting was devised that would provide clamps on all sides of the mold to prevent any leakage of gel. The molding apparatus consisted of a standard clipboard with one half inch cut from each long side. The glass plate mold was then placed so that the bottom edge was securely clamped by the metal clasp on the clipboard. Two Bulldog clamps were then used to clamp each side of the mold to the board. This arrangement proved to be a secure, leak-proof gel casting apparatus. The assembly was then secured to a ring stand in order to keep it in a vertical orientation throughout the polymerization of the gels.

C. Gel Casting/Sample Introduction:

Electrophoresis of proteins often produces a certain amount of non-reproducibility in sample bands due to variability in gel preparation and utilization. Variation in band characteristics may be caused by numerous factors such as electric charge applied,buffer constituents and temperature. For this reason, it was determined early in the project that actual electrophoresis of proteins into the gels would not provide easily reproducible sample introduction. In addition, quantification of thin bands required special densitometry which would not only add error to the measurements but also require more complex instrumentation.

The first method of sample introduction investigated was spotting. A solution of standard protein in distilled water was prepared at a concentration of 0.1 grams of Bovine Serum Albumin per liter. Slab gels were prepared according to the proportions in Table 1 and allowed to polymerize for one hour. The gel mold was then dismantled to allow the gel slab to be removed.

Table 2 - PAG Formulation

| Constituent | Stock | Quantity |
|---|---|---|
| Acrylamide | | |
| | 30:1 solution | 15 ml |
| Bis acrylamide | | |
| Ammonium Persulfate | 3% solution | 2 ml |
| TEMED | 2.5% solution | 120 microl |
| Distilled Water | | 33 ml |

For gel spotting, standard solutions of BSA were placed drop-wise onto a freshly exposed gel surface. In theory, this spotting technique would allow known spots of various concentrations of BSA to be placed easily into the gel slab. Unfortunately, the solutions of BSA did not penetrate efficiently into the gel and most of the solution simply spread across the gel surface. This method was not utilized any further.

D. Protein Inclusive Polymerization:

In an effort to develop a method for obtaining quantitative inclusion of BSA protein in PAG, the possibility of polymerizing layers which include liquid BSA in the mixture was investigated. By mixing the sample into the monomer solution prior to polymerization, it was expected that all of the BSA in the mixture would be included in the final gel. This proved to be a simple method which allows for quantitative analysis of results and lowers the possible errors due to sample introduction.

Since it is preferred that experiments provide data for several levels of BSA concentration, a method for molding gels consisting of a series of layers was perfected. It was possible to mix stock solutions of each constituent to be mixed in each layer. The proportions of gel constituents were

based on published proportions for preparation of large amounts of gel which were scaled down to allow mixture of 5 ml total aliquots of gel.

The required initiator and accelerator solutions could then be mixed just prior to their use in order to maintain consistent activity for these unstable constituents. Prior to each molding of PAG, solutions of 3% Ammonium Persulfate and 2.5% TEMED were prepared. Stock solutions of Acrylamide:Bisacrylamide were prepared by dissolving carefully weighed amounts of each constituent in distilled water. The solutions were then carefully filtered in order to remove any solid matter or large polymerized species. The filtration was accomplished with a Buchner funnel. A supply of 100 mls of this stock solution was sufficient to prepare approximately 20 four layer 9% gels.

In order to prepare layered protein inclusive gels, it was necessary to prepare each layer mixture, add the required initiator and accelerator, pour the liquid into the mold, and allow the material to polymerize prior to pouring of the next layer. If the initiator is added to all layer mixtures prior to pouring of the first layer, all would polymerize before pouring into the mold; thus each mixture received the required amount of initiator and accelerator just after the previous layer had polymerized sufficiently to prevent intermixing.

Each layer was mixed to a final volume of 5 mls. This was accomplished by reducing the required distilled water for each layer by the amount of BSA stock solution added. The acrylamide, BSA, and water were mixed together in clean 50 ml beakers. TEMED and Persulfate solutions are added to each mixture and stirred just prior to pouring into the mold.

Table 3 - BSA Inclusive Polymerization

| Conc.BSA(g/l) | 0 | .01 | 0.02 | 0.04 |
|---|---|---|---|---|
| Conc.BSA(ng/mm$^2$) | 0 | 1.0 | 2.0 | 4.0 |
| Distilled Water(mls) | 3.3 | 2.8 | 2.3 | 1.3 |
| 30% Acryl:Bis Stock(mls) | 1.5 | 1.5 | 1.5 | 1.5 |
| 0.1 grams/liter BSA Stock(mls) | 0 | 0.5 | 1.0 | 2.0 |
| 3% Ammonium Persulfate(mls) | 0.1 | 0.10 | 0.10 | 0.10 |
| 2.5% TEMED(mls) | 0.12 | 0.12 | 0.12 | 0.12 |
| TOTAL VOLUME | 5.02 | 5.02 | 5.02 | 5.02 |

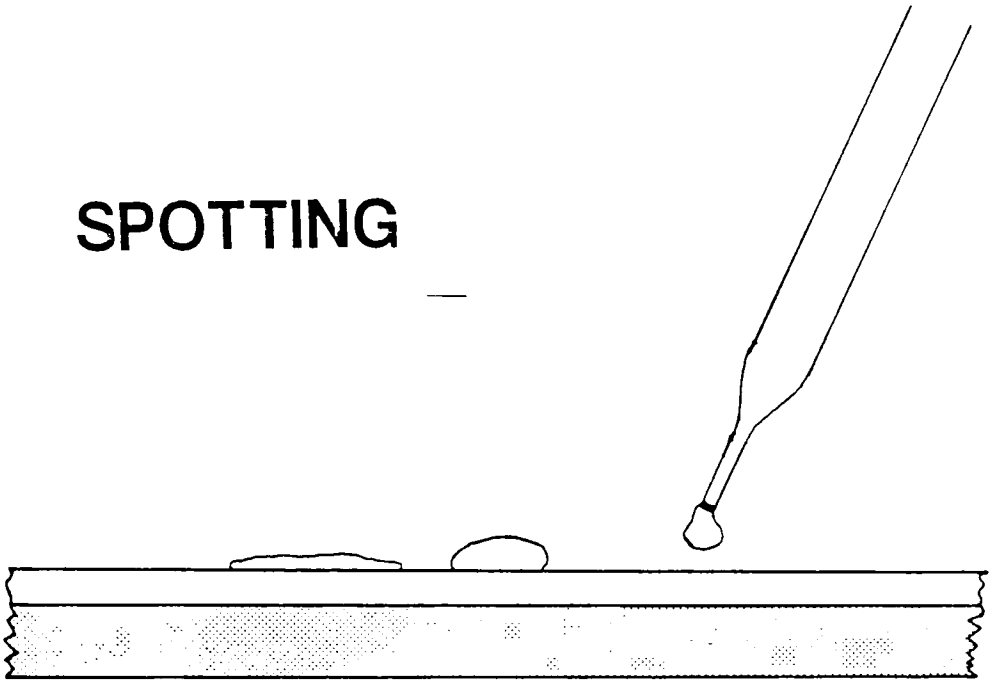



Figure 2. Gel Spotting Diagram

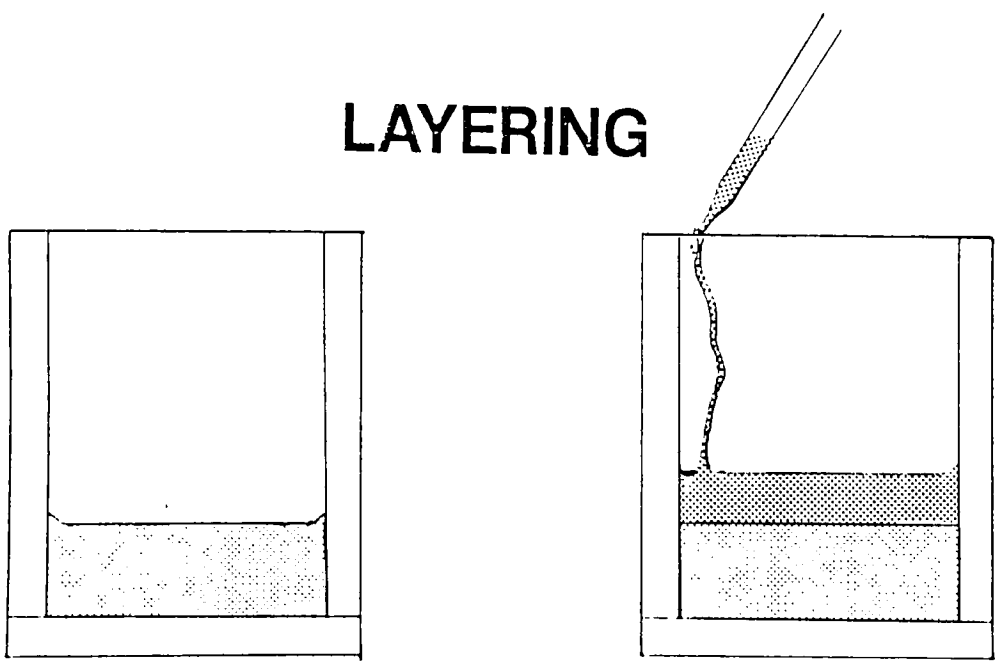
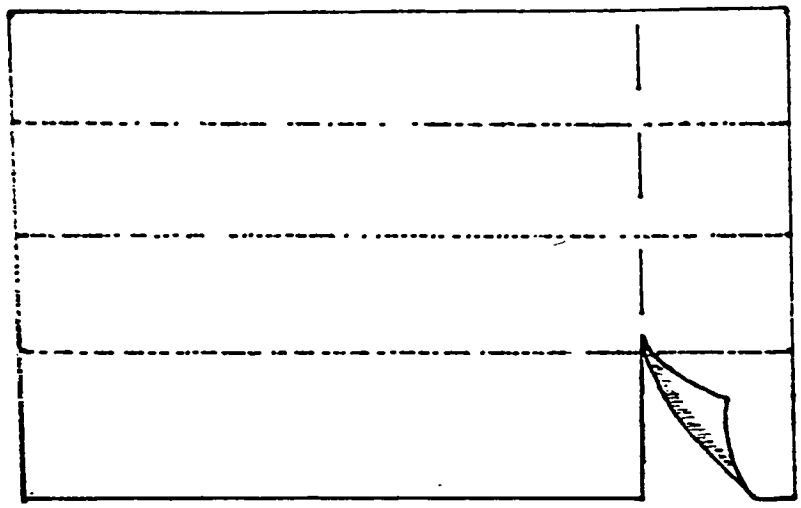
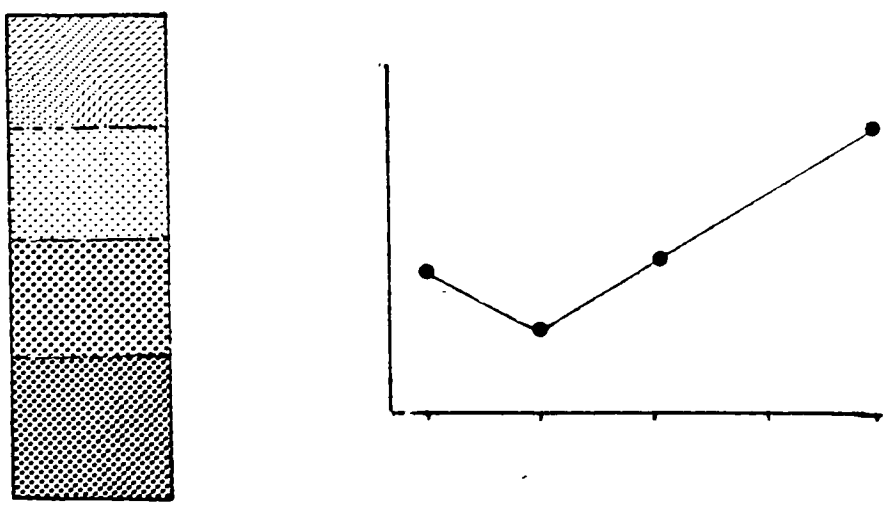



Figure 3. BSA Inclusive Polymerization Diagram



Full Polymerized Gel and Section



Stained Section and Density Graph

Fig. 3b - BSA Inclusive Gel Diagram

The layered gel process results in a solid sheet of PAG which has large, regular, rectangular sections of each BSA concentration. The sheet may be sliced after polymerization so that strips of the gel can be stained by various treatments.

E. Gel Storage:

Polymerized polyacrylamide becomes hard and brittle when allowed to dry and it was necessary to investigate methods of storing stained gels. Any gel storage method must provide a reasonable moisture seal to keep the gels soft and pliable. An attempt was made to store a test gel between sheets of aluminum foil which was carefully folded along all sides to seal the gel within. Unfortunately, this method did not provide a sufficient moisture seal, was easily torn, and the foil would stick to the surface of the drying gel.

It was found that the gels could easily and simply be stored in sealable, clear plastic bags manufactured by the Dow Chemical Company which are marketed commercially as Zip-Loc food storage bags. Gel samples, after staining, were rinsed in distilled water and then placed in the Zip-Loc bag. A small amount of distilled water was placed in the bag to provide moisture during storage. Air was then removed by laying the bag on a flat table top and gently sweeping the

surface with the fingertips before sealing. The sample bags were then stored at approximately 4 degrees Centigrade until needed for measurements or other treatments.

F. Coomassie Blue Dye Staining:

Dye staining was performed on gels in order to provide a standard to which silver stained gels could be compared and in order to test sample introduction methods such as spotting and protein inclusive polymerization.

A single dye stain was selected as representative of standard dye stains used in many labs. The most common stain cited in the literature was Coomassie Brilliant Blue R-250 (CBB). A solution of CBB dye stain was prepared and used according to the method published as part of Merril, et. al.[31] Coomassie Brilliant Blue R-250, EP grade powder was obtained from BioRad Corp.. The dye solution prepared consisted of 0.1% CBB in a solution of 50% methanol and 12% acetic acid. Gels to be stained were placed in a Pyrex baking dish and covered with the CBB staining solution. The gels were kept in this solution overnight. The stained gels were then removed to a clean Pyrex dish and covered by a destaining solution of 10% ethanol and 5% acetic acid. This solution was changed to provide a total of three treatments in the destaining bath for durations of 3 hours each.

G. CBB Linearity Test

A layered BSA-inclusive gel was prepared and stained with Coomassie Brilliant Blue R-250 as described earlier in this document. Density readings were taken over a large area of each layer using and average density values were plotted in order to display the relationship between BSA concentration and density.

H. Silver Staining Procedures:

The general procedure for silver staining proteins in PAG was based on and performed essentially as described by C.R. Merril, et. al..[32] The general silver stain used as a starting point was prepared directly from the instructions described in this publication.

All treatments during silver staining were performed in thoroughly cleaned, clear, Pyrex glass baking dishes. Glass dishes are required so that silver does not react with the container's inner surfaces.

The solutions required for silver staining were prepared as shown in Table 3. All solutions were made with fresh distilled water and were stored in clean volumetric flasks.

All of the solutions were prepared in advance and stored at room temperature with the exception of the two solutions containing formaldehyde. The Silver/Amine solution was prepared with all components except formaldehyde. The 0.5 milliliters of formaldehyde were added to the Silver/Amine solution just prior to its use in an effort to increase stability. The developer was mixed as a 3% sodium carbonate solution which was stored until approximately ten minutes before use. At that point, the benzotriazole and formaldehyde were added to produce the working solution. A benzotriazole stock solution of 0.06 grams per 100 milliliters was prepared so that additions of benzotriazole could be made by liquid measure.

Table 4 - Silver Stain Solutions

| Solution | | Constituents |
|---|---|---|
| Fixative | 50% | Methanol(absolute) |
| | 12% | Acetic Acid |
| Wash | 10% | Ethyl Alcohol(denatured) |
| | 5% | Acetic Acid |
| Bleach | 0.5% | Potassium Ferricyanide |
| Water Wash | | Distilled Water |
| Silver/Amine | 0.2 | grams Silver Nitrate |
| | 0.2 | grams Ammonium Nitrate |
| | 0.06 | milligrams Benzotriazole |
| | 0.5 | milliliters 37% Formaldehyde |
| | in 100 | milliliters distilled water |
| Developer | 0.5 | milliliters 37% Formaldehyde |
| | 0.6 | milligrams Benzotriazole |
| | in 200 | milliliters 3% Sodium Carbonate |
| Stop | 1% | Acetic Acid |

Table 5 - Silver Stain Procedures

| Solution | Time |
|---|---|
| Fixative | 10 Minutes |
| Wash | 2 x 5 Minutes |
| Bleach | 5 Minutes |
| Water Wash | 3 x 20 Seconds |
| Silver/Amine | 20 Minutes with Illumination |
| Developer | 1 x 1 Minute with Illumination |
| | 1 x 5 Minutes with Illumination |
| Stop | 5 Minutes |

All silver staining treatments were made using solutions which had been cooled in an ice bath to 20 degrees Centigrade. This was especially important since the ambient temperature of the lab varied between 18 and 32 degrees Centigrade during the experimental work.

Early silver staining trials used a high powered tungsten lamp to provide the illumination required during the Silver/Amine and Developer steps. These lamps were obtained from the Photographic Chemistry Lab and consisted of a lamp in a housing with a large reflector. This assembly was mounted on a movable stand. The use of these lamps was quickly discontinued because of the inherent non-reproducibility of the illumination. The illumination intensity could easily vary by small changes in placement or angular adjustment of the lamp. In addition, the lamps tended to produce excessive amounts of heat which was detrimental to both experiment and experimenter.

A permanent, immobile, standard light source for use in the experiment was constructed using readily purchased materials and mounted to the underside of a shelf over a countertop. The assembly consisted of an electrical cord with a grounded plug, a standard porcelain ceiling socket, and a 150 watt indoor-outdoor floodlamp bulb(Sears S-9N-PAR-38F). The distance between the mounted bulbs front surface and the

countertop was 23.5 inches.

Silver staining began by removing the gel from between the glass mold plates and carefully placing it into a Pyrex baking dish containing approximately 50 milliliters of fixative solution. An additional 50 milliliters of fixative was then added to cover the gel and to provide sufficient depth to prevent exposure of the gel to the air.

Agitation of gels began with the fixative and continued throughout the entire staining process. After each solution was added to the tray containing a gel, the tray was tilted to provide agitation every twenty seconds. In order to provide uniformity, the tray was lifted on a different side each twenty seconds. This method provide flow of the solution in alternating directions and prevented buildup of oxidized chemistry or warmer solution at any point in the tray.

## I. Silver Stain Repeatability

Over the course of the investigation, several gels were stained using the published Merril stain as a starting standard. These gels were often stained concurrently with experimental staining formulations in order to act as experimental controls. These gels also provide an indication of the standard staining procedures repeatability over the

course of the investigation.

## J. Intermediate Stain Characteristics

In order to examine the effects of intermediate staining procedures, density readings were made of gel at several points during the standard staining process. Readings of untreated gels, gels silver stained through the Silver/Amine step,and gels stained through the first development step were recorded for comparison to experimental stain results.

## K. Silver Stain Component Variation

Experimental results indicated the need for a study of fixation time variation on silver staining. By using the original published stain, it was possible to vary only the first fixation step in order to investigate this factor. A single sheet gel was fixed for the recommended ten minutes. One third of this slab was then sliced off and transferred to another dish to continue the staining process. A second third was removed and staining continued after 70 minutes in the fixer had elapsed. The final third was processed after a total of 130 minutes in the fixative. All three slices were processed through the remaining stain treatments as soon as they were removed from the fixative.

The original thrust of this thesis work was to develop a modified silver stain which would incorporate the advantages of concurrent chromogenic dye formation in order to produce a high sensitivity. The first requirement for this was to substitute new developing agents for the formaldehyde that is normally found as a developer in the silver stain.

The first developer tested was paraphenylenediamine because of its important usage in modern chromogenic development systems. Gels were stained with solutions containing developing agents which deviated from the published formulation by removal of formaldehyde and addition of PPD. The developer was mixed using 0.5 grams per 200 milliliters of 3% sodium carbonate with 0.6 milligrams of benzotriazole.

The results of PPD substitution indicated that substitution with other known photographic developing agents might provide greater selectivity. Therefore, gels were developed using solutions substituted with 0.5 grams hydroquinone, and then 0.5 grams metol. For all developer substitution experiments, the general procedure was identical to that described for the original formaldehyde developer stain.

It was also noted that the silver/amine treatment as described by Merril included a small amount of formaldehyde.

An experiment was run in which the content of the silver/amine solution was varied in order to observe the effects of the formaldehyde in this step. A gel was molded and treated as described by Merril with the exception of the silver/amine solution. Individual samples cut from the original slab just after the water wash step were treated in silver/amine solutions containing 0.25 (0.5 x Normal), 0.5 (Normal), 1.0 (2 x Normal) formaldehyde. After the samples were removed from each silver/amine bath, the process was then identical for all samples. In this way, each sample was treated the same with the exception of the silver/amine treatment which varied only in formaldehyde concentration.

L. Reduction of Background

A major detractor inherent in the use of the standard Merril silver stain was unwanted background density. This problem tended to be quite noticeable in gels made during the course of this investigation. It appears that for some as yet undetermined reason, the gels produced higher background levels than literature reports.

Since the background densities formed during silver staining were anticipated to consist of some form of silver metal, the use of existing photographic silver treatments was deemed appropriate.

M. Gel Cross Section Analysis

Several gel samples were cut to reveal cross sections. Cross sections were observed in CBB stained, silver stained and silver stain background density samples. Visual examination of cross sections provided data on stain depth of penetration for several steps during the silver stain process and comparison to CBB dye stains.

N. Density Measurement Technique:

Many literature references cite microdensitometric methods for reading density values from stained gels. These researchers are compelled to use microdensitometry due to the nature of the electrophoresed samples which yield small lines of density after staining. Microdensitometry is desirable for clinical researchers because a density chart can be obtained which is easily interpreted for diagnostic purposes. However, the use of complex measurement systems can add errors that can often be avoided by use of simpler methodologies.

Since a simple method was found which produced large areas of a single protein concentration in PAG, it was possible to use uncomplicated macrodensitometric methods in

this study. This allowed simple procedures with easily accessed equipment for density measurements.

All densitometric readings were made using a MacBeth TD-104 digital densitometer located in the Imaging Science sensitometry complex. All readings were made after carefully zeroing and checking calibration of the densitometer set with a 2 mm aperture and visual(amber) filtration. It was reasoned that visual filtration was needed for density measurement because most electrophoresis gels are interpreted by human observers and therefore the human optical system must be accounted for in determining staining sensitivity.

Density measurements were facilitated by the storage of gels in the clear plastic bags. It was possible to read densities directly through the plastic. The gels did not have to be removed and since the gels were soft and moist, the bags also protected the densitometer from water damage.

Early density reading were calculated by averaging a minimum of eight readings. Later work on the silver stain produced gels with some dependance on density formed with gel position. For these gels, a series of approximately twenty density readings were taken across the gel section. The readings were then graphed and a central, flat portion was used to estimate a density for each layer.

The information collected in this thesis was mainly in the form of density measurements. These measurements were made using equipment which had a certain level of intrinsic repeatability and accuracy. In addition, the non-uniform nature of the densities formed in the samples added to the problem of reliable data collection. In order to assess a method which would allow reasonably repeatable and accurate measurements to be made, several sampling plans were tested. The general statistics rule of thumb requires thirty samples to be made in order to provide reliable values. Due to the large number of measurements that were needed during this study it was determined that a reduced sampling plan would be needed.

In order to find a reasonable sampling plan a sample of stained gel was used as a standard. Ten sample measurements were made of each stained band using the MacBeth TD-50 densitometer (visual filter and 2 mm aperture). It was then possible to manipulate the data so as to provide single values for the density of each band.

A simple averaging of all data values was used. Since the stained gel often had dark spots or scratches, it was apparent that individual aberrant high values would disproportionately affect the simple average. For comparison,

averages of the middle eight sample readings were made; this eliminated both the highest and the lowest sample values from influencing the mean.

A comparison of the simple average and the mid-point average values showed very close agreement for up to two significant figures. Because of the nature of the measurements, it was determined that the values for analysis should contain no more than two significant figures. Both methods provided reasonably repeatable values at two significant figures.

# RESULTS

The following pages include graphical results of experimentation described in the Methods section of this document. All of the data presented used BSA inclusive polymerization techniques which were developed as part of the thesis work.

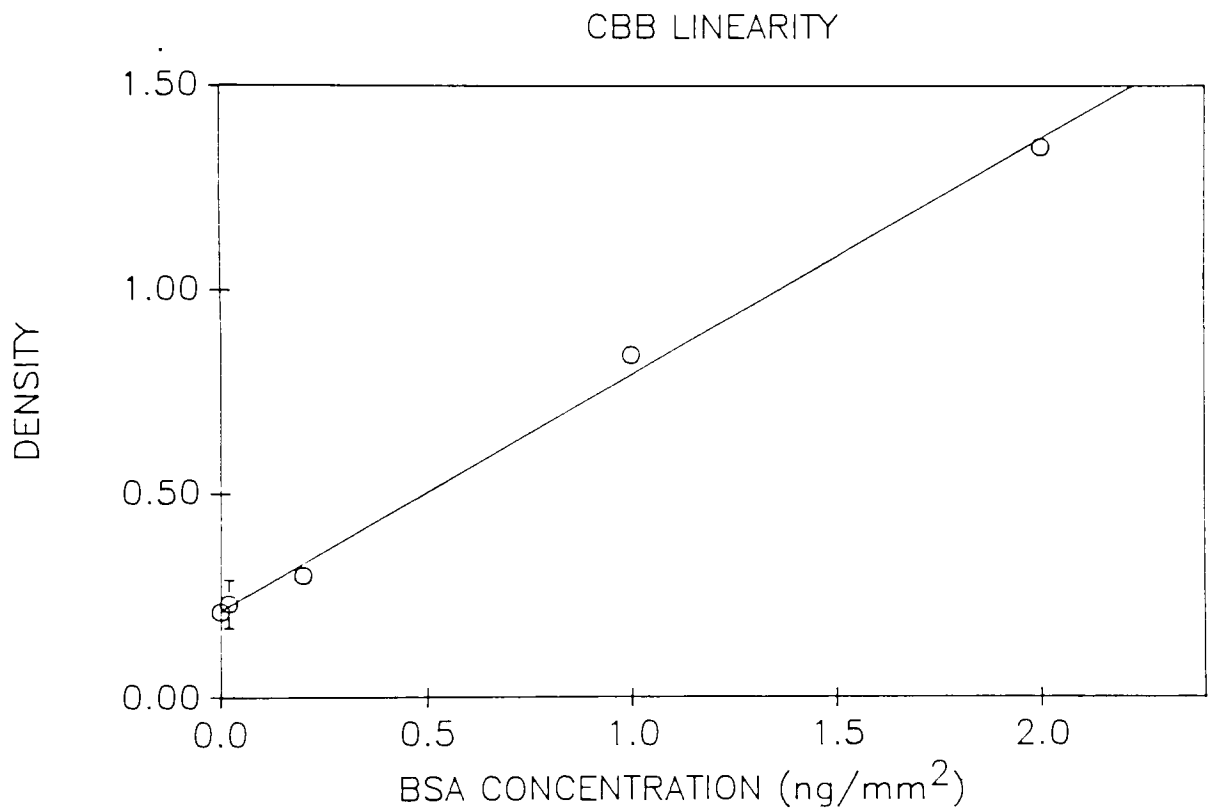



Figure 4. Coomassie Blue Stain Linearity

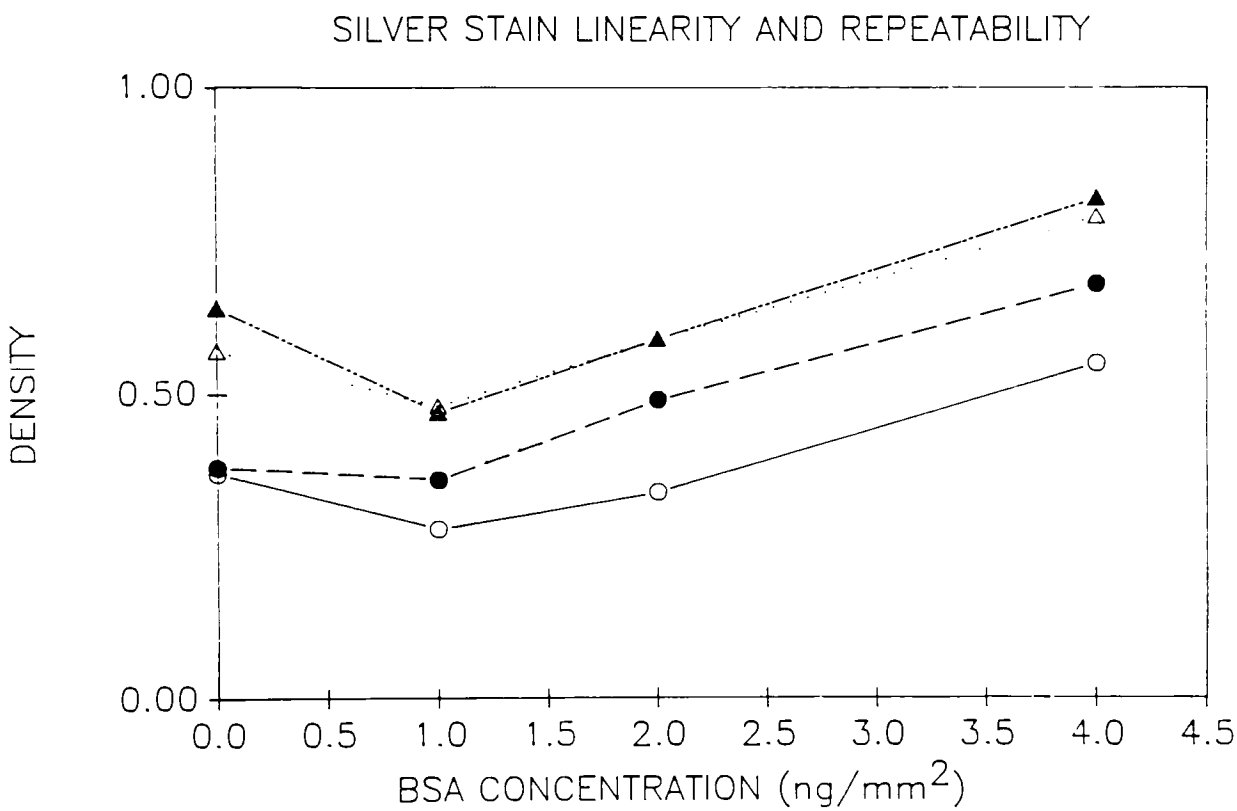



Figure 5. Silver Stain Linearity and Repeatability

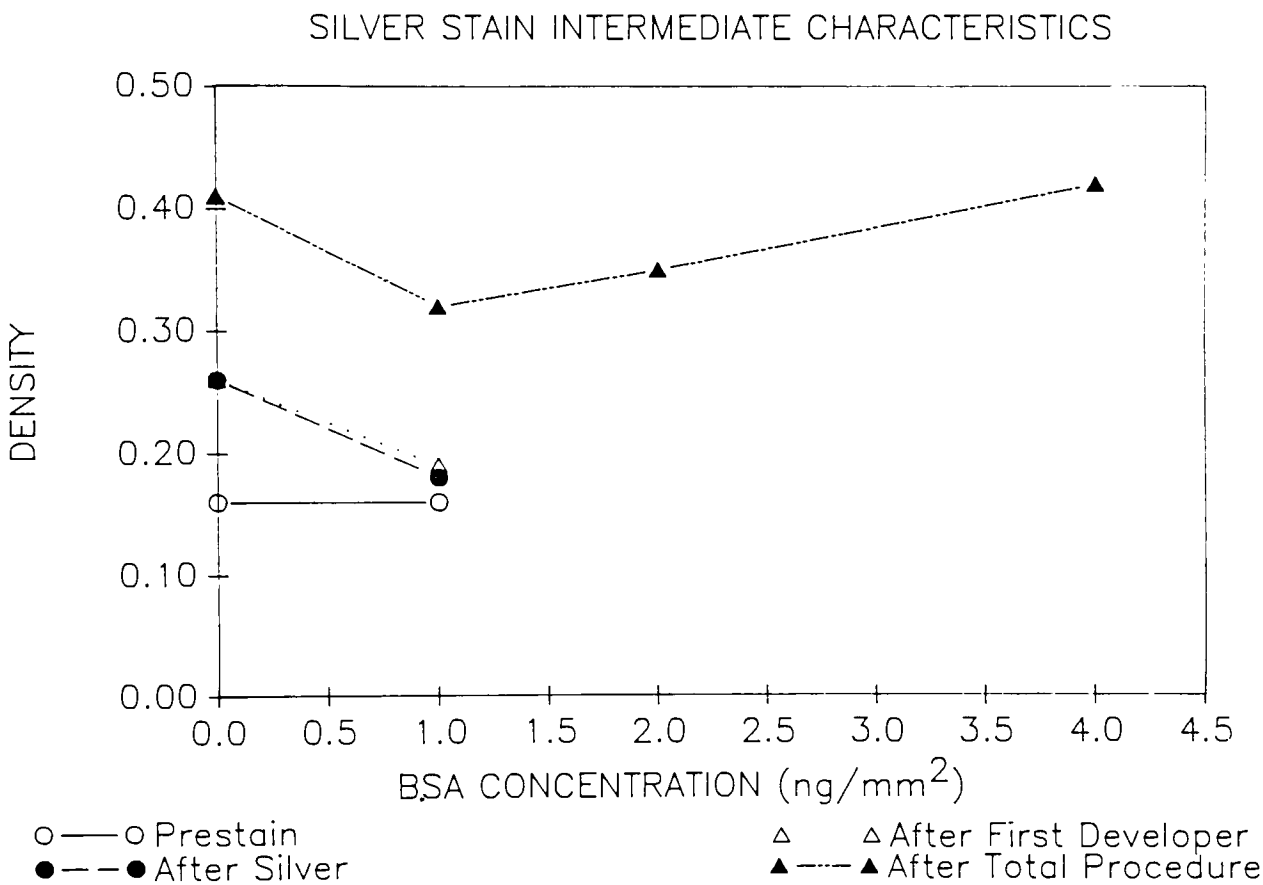



Figure 6. Silver Stain Intermediate Characteristics

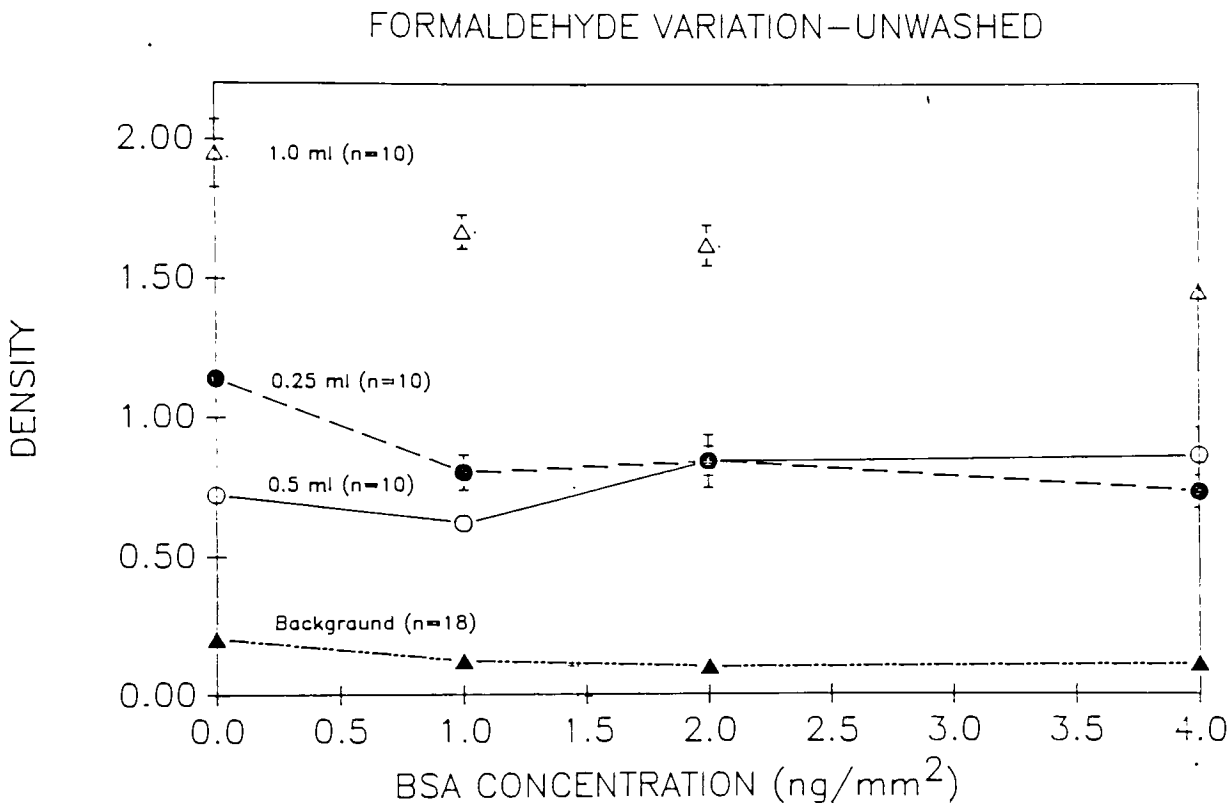



Figure 7. Silver Stain Formaldehyde Variation (Unwashed)

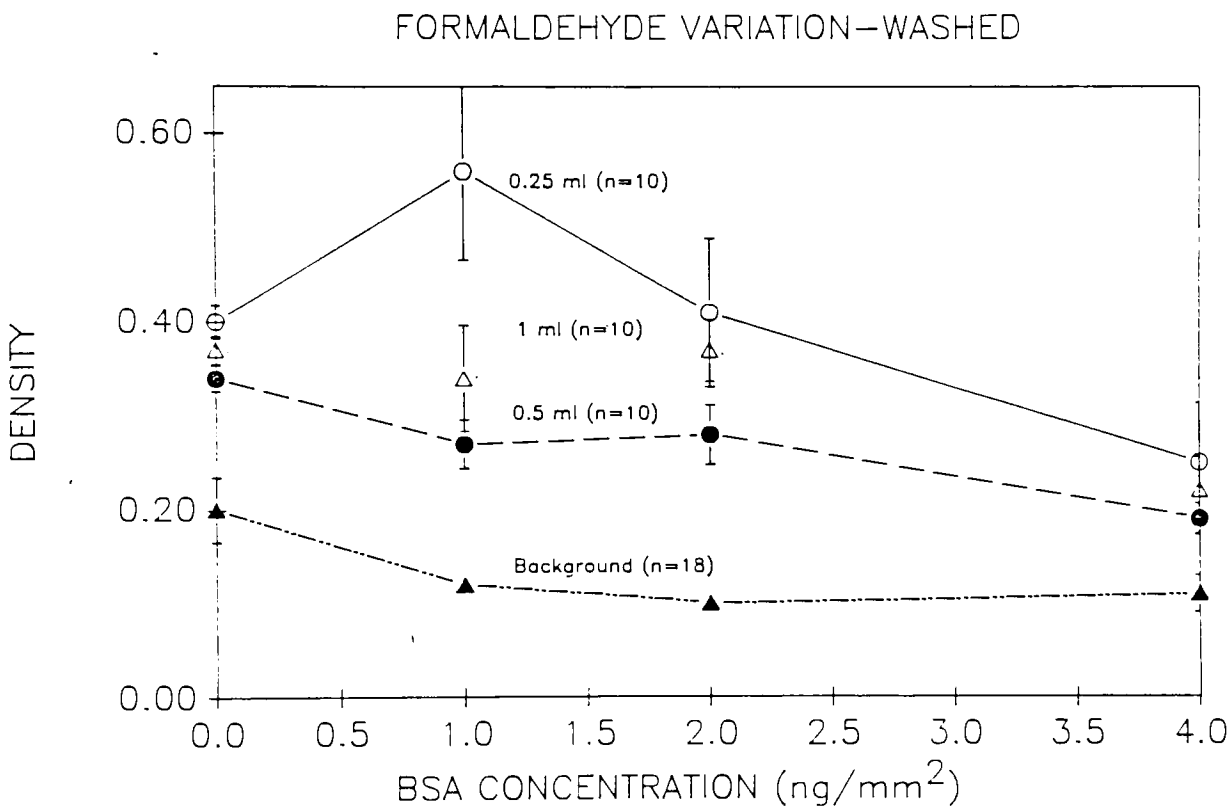



Figure 8. Silver Stain Formaldehyde Variation (Washed)

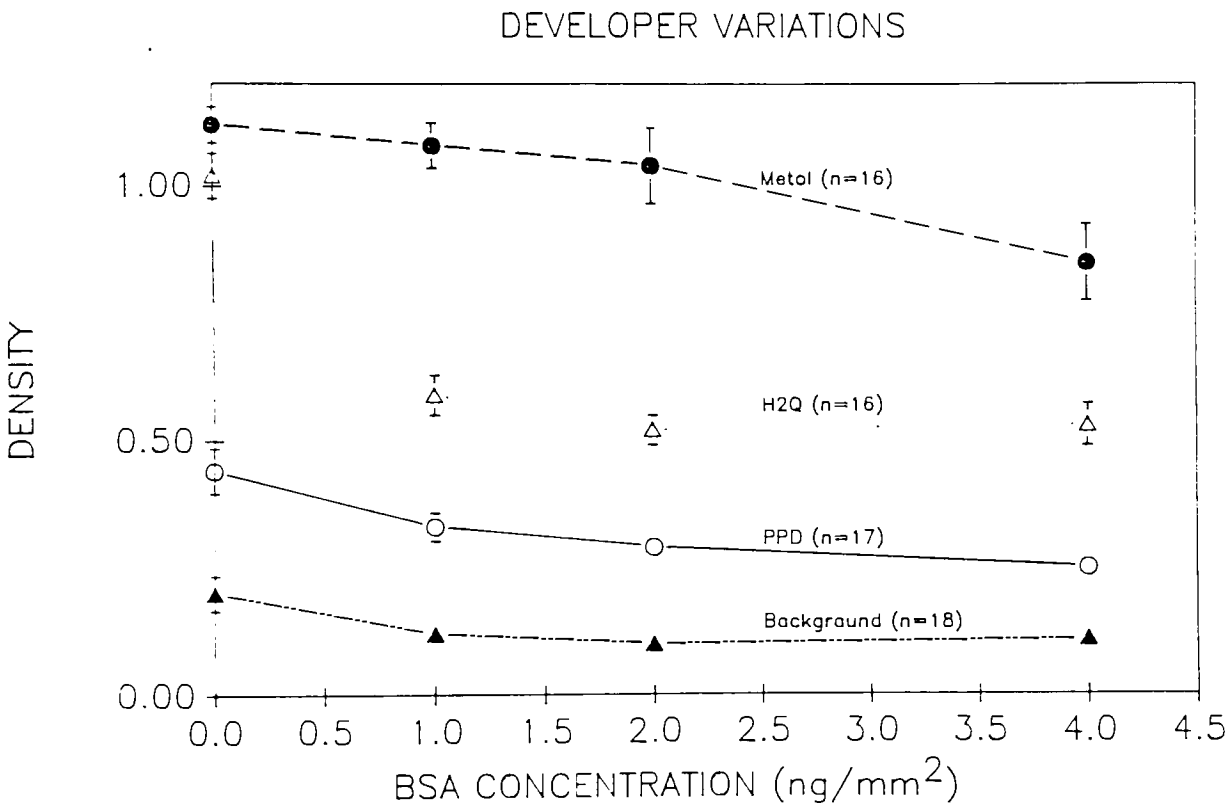



Figure 9. Silver Stain Developer Substitution

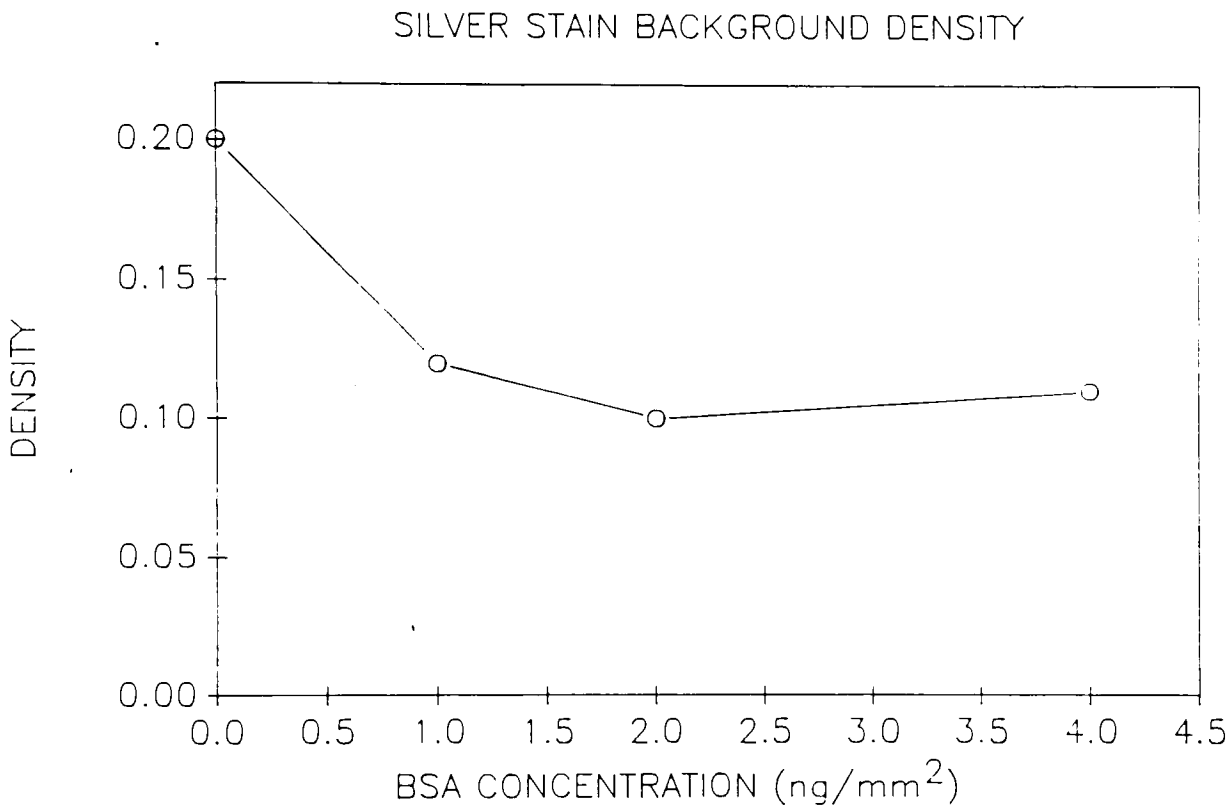



Figure 10. Silver Stain Background Density

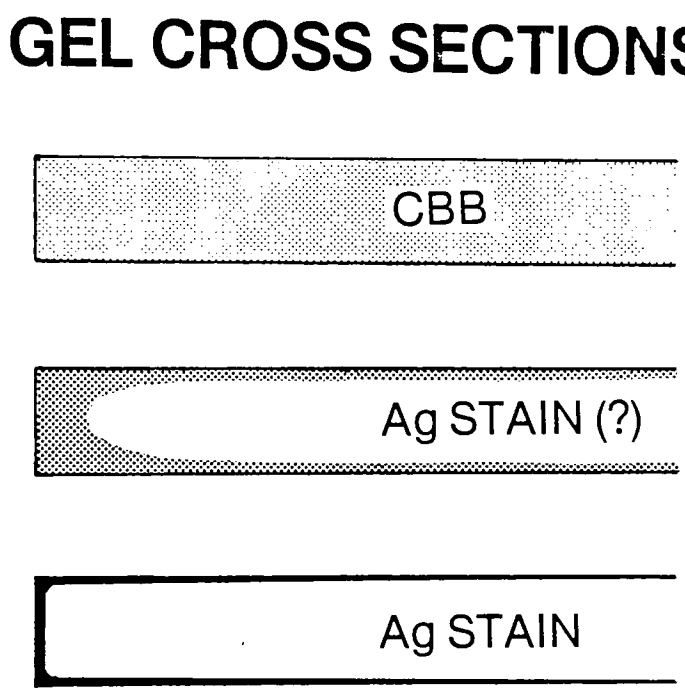



Figure 11. Gel Cross Section Diagram

# DISCUSSION

The original intent of this study was to investigate the possibility of increasing the sensitivity of existing silver staining techniques by variation of the developing agent used. It was anticipated that concurrent chromogenic dye formation could be used to accomplish this increase in sensitivity . Changes in the direction of the research were made as the experimental results indicated.

The investigation indicates several key results about the silver stain. Each experiment provided information regarding changes to the formulation of the silver stain as published. The major investigation centered on variation of the developing agent used and changes on the formulation of the silver/amine treatment.

## A. Coomassie Blue Staining

The early use of CBB in the study not only provided data on organic stain linearity and sensitivity, but it also yielded a convenient method for testing the experimental design for gel production. It was possible to experiment with methods for introducing bovine serum albumin (BSA) into the gels and, using CBB, obtain a rapid indication of the merits of each method.

The use of CBB on early layered gels showed that it was possible to polymerize BSA directly into uniformly distributed layers. The gels stained with CBB were extremely uniform which indicated a high confidence that BSA was being distributed uniformly in the polymerization of the gel layers.

A layered gel was stained with CBB in order to provide a standard for comparison between dye staining and silver stains. The stained gel showed a deep blue color in areas containing BSA in concentrations above 2 nanograms/sq. mm.. The gel sections below this concentration had a lighter blue color that was still visually detectable. The most significant property observed was the linearity of the dye stains. The gels stained with CBB showed linear density formation between zero and 20 nanograms/sq. mm. of BSA.

The linearity seen in the organically stained gels indicates that the mechanism of staining is directly related to the number of BSA molecules present. A constant number of dye molecules will attach to each BSA molecule. This observation is consistent with the theory that these dyes stain by attraction between charged groups on the sample

molecule and an oppositely charged dye molecule.[33] Since each BSA molecule in the sample should have an equal number of charged groups a linear deposition of density would be expected.

B. Merril Silver Stain

The first investigations into silver staining were mainly attempts to reproduce the process as published by Merril et al.[34] After several attempts, practices and general procedures evolved which became standards for silver staining experimentation during the study.

Many conditions of the published silver stain were not reproduced in this study due to equipment availability, unspecified factors, and experimental design. Two important factors which were not matched to those described in the silver stain published by Merril and his coworkers were illumination type and method of sample introduction.

The illumination source described in the published silver stain is a fluorescent grid lamp. This type of source has a spectral output which is different than the tungsten source used in this study. A fluorescent source, unlike a tungsten bulb, has a large ultra-violet component. It would be

reasonable to assume that this ultra-violet radiation would result in a more sensitive system since most organic molecules have a high UV absorbance. However, Dr. Merril's report describes these lamps positioned such that the radiation must pass through a thick glass tray and then through the liquid in the tray and finally through the polyacrylamide gel itself. All of these media have significant absorption in the UV portion of the spectrum.

The spectral sensitivity of the silver stain is a factor that was not directly addressed in this study due to the relative complexity of the experimental setup required. It is hoped that this question will be investigated by others in the future.

Since most of the research in silver staining techniques is published by those who work routinely with electrophoresis of samples, it is not surprising that this would be the preferred method of sample introduction cited. However, electrophoresis of samples introduces many additional steps and equipment requirements. Also, a sample which has been introduced to the gel by electrophoresis does not provide spots of directly known concentration and area. Spot samples also require microdensitometers for measurement of spot densities which requires careful alignment and calibration.

For this study, an alternate method of sample introduction was designed which simplified many aspects of the experimental investigation and data collection. By directly adding the BSA to standard volumes of gel solution prior to polymerization, it was possible to produce large areas in the gel which contain known concentrations. These large areas allowed the use of conventional densitometers for data collection. This unique method of sample introduction and densitometry does not make it possible to compare results directly to the published literature, but it does provide a new method for stain analysis.

C. Merril Stain Repeatability

The Merril stain was used as a control standard during the course of the experimentation. This provided data demonstrating the repeatability of the Merril stain under existing laboratory conditions over several months. This data provides indication of the effects of ambient temperature (which were not well controlled in the lab used), multiple process solution mixtures, and other variables.

A graph of the control data shows that there was a significant variation in the absolute density for each sample concentration between individual runs. However, the slope of

the densities produced within each control run was very consistent. The slope of all curves showed little variation from 0.29 density units per nanogram/$mm^2$ BSA in the region between 1 and 4 nanograms/$mm^2$.

D. Silver Stain Component Variation

Early observations indicated that the unwanted background fog first was seen during the silver/amine treatment. This led to investigations involving both the concentration of the formaldehyde used as well as the effect of a post silver/amine treatment wash. The experiments were accomplished by splitting a large single gel into smaller parts, each of which received different treatments following the split.

The results indicate that the amount of formaldehyde present in the silver/amine treatment produce a direct effect on both background and image density. Gels which were treated with higher than recommended concentrations showed large increases in density; however the effect was not always linear. The only concentration which exhibited greater density in the BSA containing gel then that in the blank was 2.5 ml/l. This indicates that the stain selectivity is closely tied to the concentration of formaldehyde present. It is possible that an equilibrium state drives the selectivity.

E. Developer Substitution

The original intent of this study was to improve the staining process by substitution of developing agents. It was hoped that this would lead to the ability to include chromogenic dye formation as part of the density formed in the stain.

Systematic replacement of formaldehyde in the developer solution with other well known photographic developing agents proved to be ineffective. The densities formed by these substituted developers were completely non-selective. The results for all developers tried were uniform density formation over the entire gel without regard for BSA content.

At first it appeared that these developers were providing a mild negative stain. However, closer examination revealed that the negative stain was a remnant of the silver/amine background fog.

When the background negative fog was covered by a uniform density the overall effect was to exhibit negative staining. For improved tracking of the developer effects, it was necessary to subtract values for approximate background fog from the final density of each layers.

F. Background Stain

By using the general procedures and solutions described the Merril publication, it was possible to produce dark brown-black density in gel layers containing BSA. However, several unwanted background effects were apparent. The first of these was simply the build-up of stain density in areas with no BSA. This was attributed to the limited selectivity of the stain and had been reported by many researchers as a major limitation to silver staining.[35]

The second type of background effect observed during the investigation was a cloudy, opalescent fog which appeared as a negative image. Gel layers with no added BSA had a large amount of cloudy background which decreased as BSA was added. Layers with 2 ng/sq.mm or more had no visually detectable diffusion of light. The cloudy scattering density forms during the silver/amine treatment and was detected in gels that were completely processed as well as in the test gel for which processing ended after this step.

This diffuse negative density was strongly observed when developing agents were replaced as part of the experimental investigation. It was noted that the density formation from the reduction of silver had virtually no selectivity with the photographic developing agents used . When these gels were

measured, the predominant trend was of the cloudy background. This left a result which was effectively a diffuse negative stain below a dense layer of reduced silver.

These observations indicate two separate interactions in silver staining. It appears that the silver/amine step causes an interaction with some species in the gel causing a substance which acts to diffuse light passing through the gel. It may be postulated that these diffusers are small particulates which form between the silver and some as yet unknown gel impurity. However, this reaction is hindered by the presence of BSA in the gel.

During the study two lots of acrylamide were used. The lot which was of higher purity showed this diffusion reaction to the same degree as the lower purity lot. For this reason, it is likely that the reaction is not with an impurity in the acrylamide but with some other as yet undetermined substance in the gel itself.

Other researchers, noting a similarly described diffuse background have determined that the concentration of silver in background and sample containing areas of gel are equivalent.[36] It has also been theorized that this background density is a silver precipitate such as silver chloride.[37]

## G. Background Bleaching

Since it appeared that the unwanted background density must be some type of silver compound, it seemed appropriate to attempt its removal by bleaching. It has been reported in the literature that some background density in over-developed gels can be removed with "photographic fixer" solutions. These solutions, containing mainly sodium thiosulfate, act as mild bleaching agents for the small silver background species.

It was then an obvious step to attempt the use of some of the other well known bleaching and reducing solutions as applied to silver halide photographic systems. It was easy to see that the cloudy background as well as the dark stained density were both affected by each of these solutions. This supports the theory that both are silver compounds. The effect on the cloudy density appeared to be quite rapid, faster than that on the stained density. It was possible to place a blank, stained section which exhibited cloudy background into these solutions and observe the clearing reaction as it migrated through the gel from the surfaces.

It was noted that after treatment with reducer to remove the cloudy background, the gel showed a slight blue coloration or tint. Upon observation of the reducer formulation in comparison to a simple photographic blue staining formulation, several similarities were noted. Both systems contained ferricyanide compounds which, in their normal photographic application, react with the silver metal. It is theorized that the blue tint is the result of insoluble silver ferrocyanides, e.g.,

$$4Ag + 4K_3Fe(CN)_6 \longrightarrow Ag_4Fe(CN)_6 + 3K_4Fe(CN)_6$$

In this application the blue tint is merely a side effect. However it may be possible to obtain an enhancement of measured or observed density by use of color filtration on samples treated to produce tint densities.

# CONCLUSIONS

Researchers using electrophoresis for identification and analysis of biological samples have adapted many existing photochemical processes to improve staining procedures. The similarities between silver staining systems and conventional silver halide photographic systems provide numerous areas for a beneficial cross-over in techniques and formulations.

Adaptation of a standard practice of photo-scientific methods to EP silver stains was accomplished by development of a method of producing standard test wedges of proteins in polyacrylamide gels. The method of protein inclusive polymerization as described provides an efficient method to systematically study of staining systems without the need for time consuming electrophoretic separations.

Protein inclusive polymerization was used to study CBB staining and variations in a published silver stain.

During the investigations pursued in this work several areas requiring further investigation were observed. The species or complex that causes the background density in silver stains has not yet been fully identified. Previous work has confirmed the presence of silver but the actual complexing species and morphology has not been determined.

Most silver stains published require one or more treatments to be performed under a bright light exposure. There is little information on the spectral sensitivity of these treatments. It is quite possible that the sensitivity of silver stains could be increased for many researchers if this factor was understood and optimized.

The use of protein inclusive polymerization also raises the question of calibration to samples separated by electrophoresis and measured with other techniques(i.e. micro-densitometry).

Other areas of silver staining that are open for additional study are background density reduction and developer optimization.

Also, as noted earlier, the original impetus for this work was the concept that silver stain sensitivity could be enhanced by adaptation of color photochemical processes to

form a silver metal and dye density in a single development step.

Recently, researchers have successfully adapted this concept of using chromogenic dye density formation to stains for histological samples inspected by light microscopy. Among these techniques are applications of color development to microautoradiography[38] and, more recently, immunogold-silver intensified stains for light microscopy.[39] These systems use the dye image to enhance visual contrast of sample structures. The development of these chromogenic dye stains for histological use validates the concept that dye forming development can be adapted to biological stain systems. Since many silver stains for electrophoresis were derived from histological silver stains the adaptation of dye formation to electrophoresis gels is feasible. The combination of dye density and silver density would increase the detection limit of silver staining systems.

The use of the Sample Inclusive Polymerization technique described in this work could prove to be an excellent tool for further research into future staining systems. The ability to measure stain response characteristics quantitatively could greatly accelerate progress toward hyper-sensitive stains.

# APPENDIX A

## ELECTROPHORESIS

Electrophoresis is a high resolution separation technique used by a wide number of laboratories involved in analysis, research and clinical diagnostics. In general, the different electrophoretic procedures differ from chromatographic methods in that voltage differences are the driving force for the movement and separation of the sample molecules. Electrophoresis has found wide use in the separation of proteins, nucleic acids and enzymes, as well as many other organic molecules.

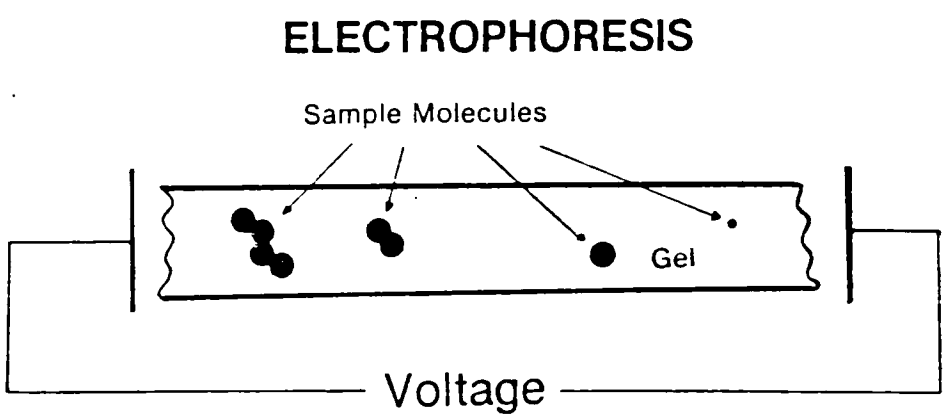



Fig. 12 - Electrophoresis Diagram

Early EP methods used sample molecules in solution through which an electric current was passed. The charge differences of the sample molecules resulted in a differential in the speed of travel in the solution and the separation was thereby effected. This "moving boundary" method had a number of problems that limited resolution and thereby usefulness to researchers. Some of the problems encountered were convection currents, heat dissipation,sensitivity, ease of sample handling and cost.

As noted earlier, the molecules are separated according to their charge, or, more specifically, their charge density (charge/mass ratio). The sample, placed at one end of the gel(or tank), begins to migrate under the influence of high voltage applied to the system. The voltage is high because the resistance of the gel/buffer system is often great. Following physical laws, the speed of the separation is dependent on numerous factors such as; sample molecule size, structure of the gel, pore size of the gel, buffer, magnitude of the voltage applied to system, resistance of the system, and numerous other system parameters. For this reason, standard "known" samples are often run simultaneously alongside unknowns in order to internally calibrate the system. This provides a basis for comparison between runs that may not have

identical conditions.

Many of the problems inherent in moving boundary techniques are minimized or eliminated by the inclusion of a rigid, non-conductive, porous media as a basic structural network. Some of the materials that have been used for this purpose include; starch gels, cellulose acetate, paper, agarose and polyacrylamide. Agarose and polyacrylamide gels have been the most popular and are now readily available in a number of commercial forms. The use of these gels, along with improved methodology, has greatly increased the resolution and applications of modern EP separations.

GELS - Polyacrylamide(PAG) and Agarose

Presently the most common medium used for research electrophoresis is polyacrylamide gel. PAG is produced by a catalyzed polymerization reaction between acrylamide and N,N'-methylene-bis-acrylamide.(See Appendix B for monomer structures). The reaction is a catalyzed free-radical reaction that can be photo or thermally initiated with the appropriate initiating compound added to the co-monomers. Since the gel is formed by free radical polymerization, there may be

significant quenching by oxygen. The reaction produces sufficient cross-linking to allow gel formation to occur with as little as 2% bis-acrylamide.[40] The relative concentrations of the monomer and co-monomer can be varied to produce gels of specific porosity. It has been found that the pore size reaches a minimum at 5% bis-acrylamide with lower or higher concentrations producing larger pore sizes.[41]
Polyacrylamide gels are transparent, can easily be made with various porosities, are relatively easy to handle and allow high resolution separations to be made.

Although PAG is often used for EP applications, there are several problems that must be considered. The monomers and catalysts required for producing the gels are highly toxic materials. The concern for the health of lab technicians and environmental factors becomes great if high volume separation facilities are planned.

The pore sizes that can be created with PAG are often too small to separate large molecules with reasonably short separation times. For these reasons agarose is sometimes chosen over PAG.

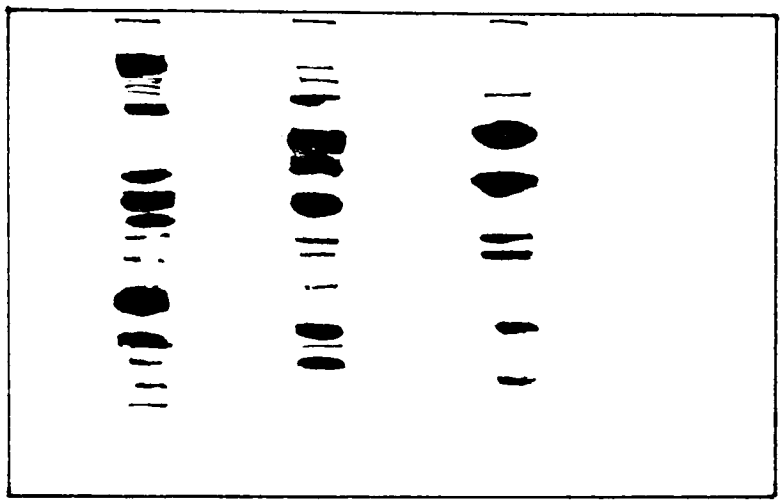
Agarose is a natural gel medium consisting of long chain organic molecules. It is prepared from seaweed and is similar in physical properties to gelatin. It is usually commercially available in powdered form and is soluble in water. The solution will remain liquid at elevated temperatures and will gel at approximately body temperature. The pore sizes of the gel are determined by the concentration of agarose used. Since no polymerization is used with agarose, there is less control and range of pore sizes obtainable. In some cases, agarose can be added to PAG to aid in control and variation of pore sizes obtained. Agarose does not have the health hazards associated with PAG and is, in fact, often used as a growth medium for plants or bacteria cultures.

## ELECTROPHORESIS HARDWARE

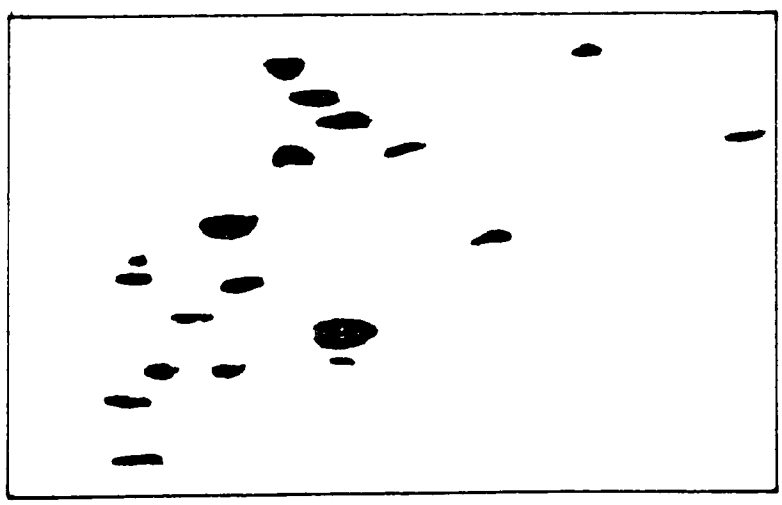
The hardware needed for electrophoretic separations is not complex. In general, all provide two major functions; they provide a safe effective source of high-voltage and some manner of support for the gel/buffer environment. There are a wide variety of style available commercially and each can differ slightly in sample orientation, size, shape or extra features.

The gel support media can be cast(molded) into various shapes and forms; molded tubes and thick or thin slabs are typical examples. The casting method is similar for any of these final molded shapes. In general, the gel components are mixed and, while still liquid, the solution is poured into the mold and allowed to set. The molds are often made of inexpensive glass tubing or spaced glass plates. When the media gels it retains the shape of the mold used. Gels that are formed in tubes are often difficult to handle and their thickness necessitates increased staining time as well as larger sample sizes. Tube gels are often used when a second dimension separation is to be used. A first separation is done in the tube gel; The gel is then split lengthwise and placed onto a slab gel for the second dimension separation.

Although slab gels have their own handling problems, they have the advantage of allowing several samples to be run side by side in the same gel. They can also be molded into very thin slabs which can be mounted on a transparent, rigid support base such as MYLAR$^{R}$ film.



Typical 1D Separation Patterns



Typical 2D Separation Pattern

Fig. 13 - Typical 1D and 2D Separation Patterns

# APPENDIX B

## CHROMOGENIC DEVELOPMENT - AgX SYSTEMS

Early color photography consisted of cumbersome attempts to record separate color records of a scene using conventional monochromatic materials. Since silver halides are only sensitive to radiation in the blue and ultra-violet region of the spectrum, color photography could only be accomplished after silver halide systems were spectrally sensitized to the entire visible spectrum. The problems of spectral sensitization began to be solved in the early 1900's after Vogel's[42] discovery of spectral sensitization of silver halides. With this first step accomplished, a practical color photographic system was not far down the road.

Very early color systems consisted of awkward exposure and projection procedures. These systems consisted of a method to make records of each color element of a scene on monochrome material and re-projecting the processed images in careful registration through an identically colored filter. Each exposure in this system was an individual record of the particular color values in the scene.

In order to create simple processes for color films, early researchers recognized the desirability of forming color dyes directly by chemical reaction inside the film itself. Chemical formation of color dyes would eliminate the need for color separation exposures and registration. In 1912 Fischer[43], and others recognized these advantages of a chromogenic dye formation system and began research toward a comprehensive system. Many problems were encountered with respect to dye formation, stability and color purity. It was most important that the dyes formed be of adequate purity in absorption in order for the system to produce a realistic and repeatable rendition of the scene. It was not until the 1930's that a commercial process was produced.[44]

In these systems, the scene is recorded on a single multi-layered film. The film consists of three photosensitive layers, each sensitized to a specific portion of the visible spectrum. The record of the scene is therefore perfectly self registering on a single film. The colored image is produced by reactions between oxidized silver halide developer and dye coupler compounds.

The chemistry of modern color development systems consists of several basic steps. An exposed silver halide

crystal, containing latent image sites, is developed by a suitable color developing agent to form silver metal and an oxidized form of the developing agent.

In standard black-and-white photographic process, this developed silver metal constitutes the final image structure. However, in color processes, this silver metal is discarded in subsequent processing steps and the dye formed becomes the final image structure.

After reduction of the silver halide into silver metal, the oxidized developing agent can then react with couplers to form color dye molecules. The efficiency of the dye forming reaction varies and is dependant on the structure of the components and the final dye formed.[45]

Color couplers can be formulated into the developer solution or incorporated into the film itself. The exact mechanism of the color dye formation is not simple and several theories involving many intermediate steps have been proposed. Experiments performed by Tong and Glesmann[46] indicate that the oxidized developer exists as a highly reactive, unstable semiquinone which quickly reacts with other developer species present to form a fully oxidized developing agent molecule.

The mechanism proposed indicates that only this fully oxidized form will react with the color couplers to form dyes.

Couplers of a similar molecular type will produce dyes with similar color characteristics and several groups are commonly used in color photography. It is known that the resulting dye color is moderated by substituent groups on both the developing agent and the color coupler molecule.[47] It is possible to produce many different color absorption characteristics from a singe base developer-coupler combination.

The optical density of many color dyes formed by chromogenic development have been found to be ten times that of silver when light of a complementary color is used for measurements.[48] Although this is often a problem with regard to the desired sensitometric characteristics of a color system, it does indicate that high efficiency dye formation systems can be produced.

The choices for developer and coupler constituents are determined by the final dye characteristics desired. Dye color purity, stability, and immobility, are several of the important characteristics required for color photographic developer/coupler system designs.[49]

# APPENDIX X

## PROCEDURE FOR CLEANING GLASS PLATES AND GLASSWARE

1) All glassware is thoroughly rinsed in warm tap water.

2) Glass is then treated with RBS-35 glass cleaning solution; Physical action is applied to remove grease etc. as appropriate.

3) RBS-35 solution is then rinsed off of glass parts with warm tap water.

4) Acid dichromate solution is applied to cover entire surface of glassware and allowed to sit for 1 min. Glass plates were placed horizontally in a tray containing the dichromate solution.

5) Acid dichromate solution is rinsed off with several changes of warm tap water.

6) Glass is rinsed with fresh distilled water and allowed to air dry prior to use.

# APPENDIX Y

Details of Chemistries Used:

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Gel Fabrication:

N-N'-Methylene-Bisacrylamide, Electrophoresis Grade,
Fisher Scientific #O-3586, Lot 723169

Acrylamide, Bulk,
Eastman Organic Chemicals #5521

Acrylamide, EP Grade,
Biorad Corp., Cat #101, Control 20557

Acrylamide/Bisacrylamide(37.5:1), EP Purity Reagent,
BioRad Corp., Cat #161 0106, Control 24943

Ammonium Peroxydisulfate, Certified ACS,
Fisher Scientific #A-682, Lot 711128A

N,N,N',N'-Tetramethylethylenediamine(TEMED),EP Grade,
Fisher Scientific #O-4653, Lot 721561

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Staining Solutions:

COOMASSIE Brilliant Blue R-250, EP Grade,
BioRad Corp, Cat #1610400, Control 22493

Silver Nitrate from Chem Lab Stock

Potassium Ferricyanide

Acetic Acid(glacial), Reagent Grade, from Chem Lab Stock

Methanol(absolute), from Chem Lab Stock

Ethyl Alcohol(denatured), Reagent Grade

Ammonium Nitrate, from Chem Lab Stock

Formaldehyde(37%), from Chem Lab Stock

Sodium Carbonate, Monohydrate, Photo Grade, from Chem Lab Stock, Church and Dwight, Lot KSG-489

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Developers:

Hydroquinone, Photo Grade,
Carus, Lot #273

p-Phenylenediamine Dihydrochloride,
Eastman Kodak Co. #207, Lot C8C

Metol, Photo Grade,
Mallinckrodt, Lot 0981700

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Sodium Thiosulfate, Photo Grade,
Allied Chemical Corp., Lot ALH1

Benzotriazole, Practical Grade
Eastman Kodak Co. #P2759, Lot 702-1

Ammonium Nitrate,
Fisher #A-676, Lot 746577

Ferric Ammonium Citrate(Green),

Fisher, Lot 734450

Sodium Citrate,

Fisher, Lot 734451

Ethyl Alcohol, Reagent Alcohol Absolute,

Mallinckrodt #7019

Acetic Acid, Reagent Grade,

Corco Chemical Corp. #300, Lot 521207

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